AWARD NUMBER: W81XWH-14-1-0037

TITLE: Uncarboxylated Osteocalcin and Gprc6a Axis Produce Intratumoral Androgens in Castration-Resistant Prostate Cancer

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REPORT DATE: May 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
May 2016	Final	1 Mar 2014 - 28 Feb 2016

4. TITLE AND SUBTITLE

Uncarboxylated Osteocalcin and Gprc6a Axis Produce Intratumoral Androgens in Castration-Resistant Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0037

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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MI 40201

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Castrate resistant prostate cancer (CRPC) represents the final and lethal disease state in the progression of prostate cancer. CRPC patients often develop bone metastasis resulting in bone fractures and morbidity. Recently, tumor cells have been shown to activate androgen receptor signaling via multiple pathways, despite castrate levels of testosterone. One such adaptive mechanism is the "intracrine" production of androgens in the primary tumor and/or at metastatic sites by the activity of androgen biosynthetic enzymes. Recent study shows that Gprc6a/Osteocalcin axis regulates physiological androgen biosynthesis in testis. Since Osteocalcin is overexpressed in patients with bone metastasis and existence of intratumoral androgen synthesis in bone metastasis, we hypothesized that bone tumor expressed Osteocalcin can induce intratumoral androgen synthesis in bone metastasis. We show that in VCaP model system Gprc6a is expressed and overexpression of its ligand Osteocalcin in these cells leads to expression of androgen biosynthetic enzymes. This data suggest that prostate cancer bone tumors hijack Osteocalcin/Gprc6a axis for the production of intratumoral androgens via overexpression of certain androgen biosynthetic enzyme expression. Bone tumor expressed androgens promote disease progression via tumoral androgen production and androgen receptor activation.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified		USAMRMC

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1. INTRODUCTION:

Castrate resistant prostate cancer (CRPC) represents the final and lethal disease state in the progression of prostate cancer. Recently, tumor cells have been shown to activate androgen receptor signaling via multiple pathways, despite castrate levels of testosterone. One such adaptive mechanism is the "intracrine" production of androgens in the primary tumor and/or at metastatic sites by the activity of androgen biosynthetic enzymes(1).

CRPC is often characterized by disease progression in bone, and osteoblasts are known to express osteocalcin. Osteocalcin undergoes carboxylation at multiple glutamic acids, the resultant gama carboxylated Osteocalcin interacts with bone extracellular matrix associated calcium and hydroxyapatite and deposited in the bone matrix. Some Osteocalcin is released into circulation without undergoing decarboxylation and recently Karsenty`s group showed that circulating uncarboxylated Osteocalcin is a potent factor inducing androgen production in Leydig cells in testes (2). Osteocalcin binds and activates a novel cell surface receptor Gprc6a in Leydig cells to induce androgen biosynthetic enzyme expression and androgen production. However, osteocalcin is dysregulated in CRPC, with higher levels of the uncarboxylated form found in patients with bone metastasis (3). We thus hypothesize that uncarboxylated osteocalcin, expressed by osteoblasts or tumor cells, leads to local biosynthesis of androgens, thereby contributing to expansion of the metastatic deposit in bone, despite castrate levels of serum testosterone. Thus, just as the skeleton regulates fertility in an endocrine fashion, and it may also promote bone metastasis via an "intracrine" mechanism.

2. KEYWORDS:

CRPC:	Castrate resistant prostate cancer
PC:	Prostate Cancer
Gprc6a	A seven transmemberane G-protein coupled receptor
AKR1C3:	Aldo Keto Reductase 1C3
17BHSD	17 beta Hydroxysteroid Dehydrogenase
RTPCR	Real time Polymerase Chain Reaction
SCID	Severe combined immuno deficient
VCaP	Vertebral metastasis prostate cancer cell line
DHT	Dihydroxy testosterone.
RFP	Red fluorescence protein

3. OVERALL PROJECT SUMMARY:

Major Goals:

Goal 1: Demonstrate the functional Gprc6a expression in prostate cancer cells

Goal 2: Determine the clinical significance of osteocalcin, Gprc6a, and androgen biosynthetic enzymes during CRPC progression.

Major activities:

1. We generated stable VCaP cell lines expressing RFP (red fluorescence protein as control) Osteocalcin and mutant Osteocalcin using lentivirus mediated stable infections.
2. Determined the gene expression of Gprc61 and androgen biosynthetic gene expression in Osteocalcin and mutant Osteocalcin infected cells.
3. We performed intratibial implantation experiment with osteocalcin and mutant osteocalcin expressing cells
4. Determined the T and DHT levels in bone tumors

Specific objectives:

Expression of Osteocalcin forms in VCaP cells: We used a lentiviral system for expressing Osteocalcin and mutated Osteocalcin. Osteocalcin is mutated at three positions where glutamic acid residue at 17, 18 and 24 were mutated to glutamine to prevent carboxylation. Both native and mutated osteocalin was cloned into lentiviral expression plasmids. Viral stocks were infected with VCaP cells to express RFP, Osteocalcin and mutated Osteocalcin. Osteocalcin gene expression was determined using RT-PCR method. Osteocalcin and mutated Osteocalcin was expressed in VCaP cells (Figure 1). Conditioned media was collected from RFP, osteocalin and mutated Osteocalcin expressing cells. ELISA quatitiation show that Osteocalcin is secreted from the VCaP-osteocalcin cells (Figure 2). ELISA does not detect mutated Osteocalcin in mutated osteocacin expressing cells.

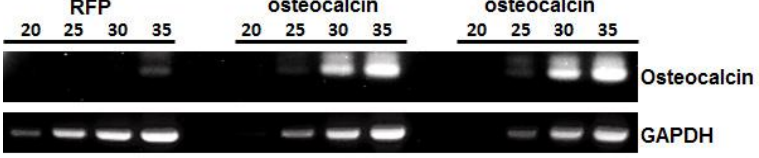

Figure 1. RT-PCR analysis of Osteocalcin in lentivirus infected VCaP cells. RNA extracted from RFP and Osteocalcin expressing cells were analyzed for Osteocalcin for indicated number of PCR amplifications. Amplified Osteocalcin and GAPDH gene products were run on the gel.

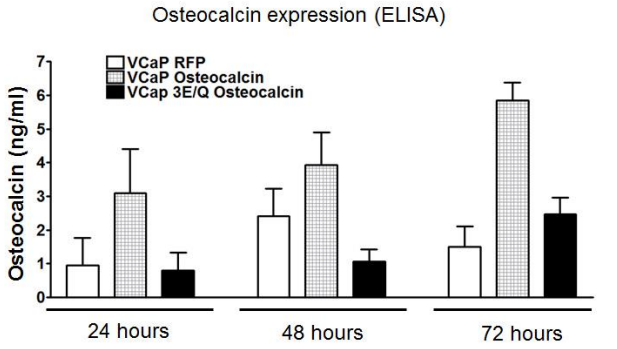

Figure 2. ELISA of Osteocalcin: Conditioned media from VCaP cells expressing RFP, wild type Osteocalcin and 3E/Q mutant Osteocalcin were analyzed for secreted Osteocalcin.

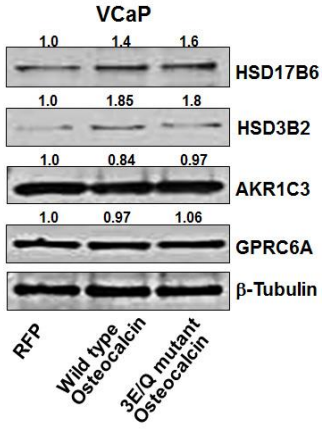

Figure 3. Westernblot analysis of VCaP cell infectants with androgen biosynthetic enzymes and Gprc6a and b-tubulin.

Determine the decarboxylated osteocalcin induced expression of androgen biosynthetic enzymes. To determine the functional Gprc6a expression in VCaP cells, androgen biosynthetic enzyme expression was determined in VCaP RFP, wild type Osteocalcin and 3E/Q mutant Osteocalcin infected cells. VCaP and VCaP transfectants express Gprc6a. HSD3B2 and HSD17B6 expression was increased in both wild type and 3E/Q mutant Osteocalcin infected cells (Figure 3).

Determine Testosterone and DHT levels in VCaP cells upon decarboxylated osteocalcin /Gprc6a activation: VCaP cells transfectants expressing RFP, Osteocalcin and 3E/Q osteocalcin cells were injected into tibae of mice. Mass spectrometric quantitation of Testosterone and DHT was performed in bone tumors. Bone tumors expressing 3E/Q osteocalcin have high production of testosterone (Figure 4), where DHT levels are higher in both osteocalcin and 3E/Q osteocalcin expressing tumors.

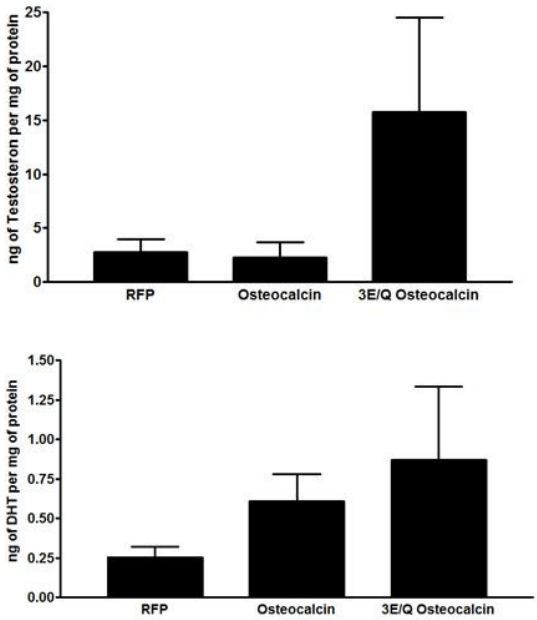

Figure 4. Testosterone and DHT levels in bone tumors

In vivo studies of Gprc6a mediated androgen biosynthetic enzyme expression: VCaP cells transfectants expressing RFP, Osteocalcin and 3E/Q osteocalcin cells were injected into tibae of mice. Bone tumors were imaged with luciferin after 14 weeks. Data show that osteocalcin tranfected cells grew slower, whereas mutant 3E/Q osteocalcin transfected cells grew larger, suggesting mutant osteocalcin promote bone tumor growth (Figure 5).

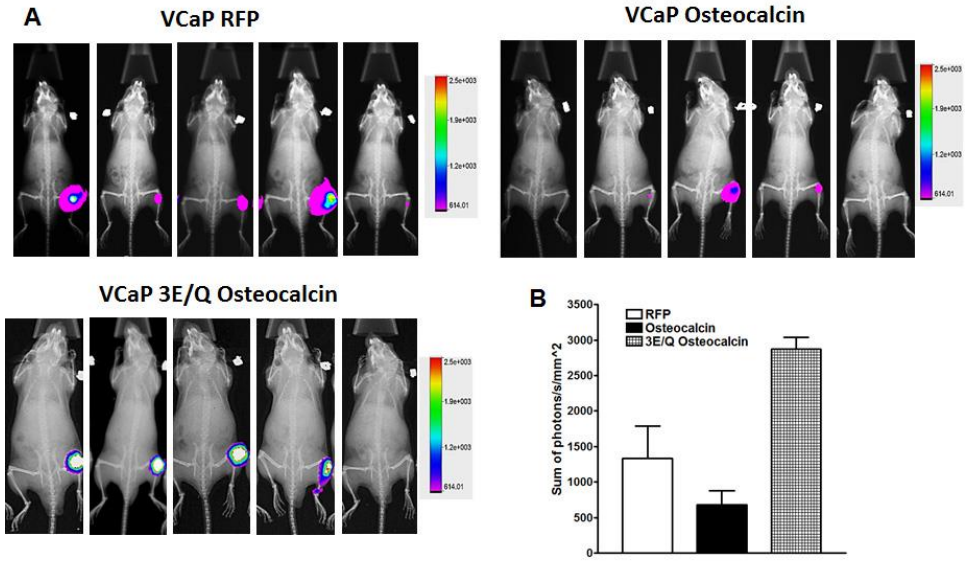

Figure 5. Bone tumor growth of VCaP osteocalcin transfectants. A. Whole body mice luciferase imaging for bone tumors. B. Quantitation of tumor growth.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Osteocalin/Gprc6a axis promotes intratumoral androgen synthesis by over expressing androgen biosynthetic enzyme expression.
- Decarboxylated osteocalcin (3E/Q mutant osteocalcin) is an inducer of bone tumor growth through activation of Gprc6a receptor.

5. CONCLUSIONS:

Our data show that Osteocalcin/Gprc6a axis is functional in VCaP prostate cancer cells. This pathway can induce intra turmoral androgen synthesis through overexpression of androgen biosynthetic enzyme expression. Together this pathway promotes prostate cancer bone metastasis and drive androgen receptor mediated bone tumor growth.

6. PUBLICATIONS, ABSRACTS, AND PRESENTATIONS:

None.

7. INVENTIONS, PATENTS AND LICENCES:

None.

8. REPORTABLE OUTCOMES:

None.

9. OTHER ACHIEVEMENT:

We developed three stable cells lines through lentivirus mediated expression of RFP, Osteocalcin and 3E/Q mutated Osteocalcin in VCaP cells.

10. REFRENCES:

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2. Oury F, Sumara G, Sumara O, Ferron M, Chang H, Smith CE, et al. Endocrine regulation of male fertility by the skeleton. Cell.144(5):796-809.
3. Gardner TA, Lee SJ, Lee SD, Li X, Shirakawa T, Kwon DD, et al. Differential expression of osteocalcin during the metastatic progression of prostate cancer. Oncol Rep. 2009;21(4):903-8.

11. APPENDICES:

None.